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Beukeboom, Leo W.; van de Zande, Louis

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REVIEW ARTICLE

Genetics of sex determination in the haplodiploid wasp *Nasonia vitripennis* (Hymenoptera: Chalcidoidea)

LEO W. BEUKEBOOM* and LOUIS VAN DE ZANDE

Evolutionary Genetics, Center for Ecological and Evolutionary Studies, University of Groningen, Groningen, The Netherlands

Abstract

The parasitoid wasp *Nasonia vitripennis* reproduces by haplodiploidy; males are haploid and females are diploid. Sex determination in *Nasonia* is not governed by complementary alleles at one or more sex loci. As in most other insects, the sex-determining pathway consists of the basal switch *doublesex* that is sex-specifically regulated by *transformer*. Analysis of a polyploid and a mutant gynandromorphic strain, suggested a parent-specific effect (imprinting) on sex determination in *Nasonia*. Zygotic activity of *transformer* is autoregulated and depends on a combination of maternal provision of *tra* mRNA and a paternal genome set. This constitutes a novel way of *transformer* control in insect sex determination implying maternal imprinting. The nature of the maternal imprint is not yet known and it remains to be determined how broadly the *Nasonia* sex-determining mechanism applies to other haplodiploids.

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Introduction

How gender is determined under haplodiploidy in the absence of heteromorphic sex chromosomes is still an unanswered question although much progress has been made in recent years. Under haplodiploidy, males and females differ in ploidy level; females are diploid and develop from fertilized eggs, whereas males are haploid and develop parthenogenetically from unfertilized eggs (figure 1). This mode of reproduction occurs in several invertebrate groups including pinworms, mites, thrips, and beetles, but occurs ubiquitously only in the hymenopteran insects (ants, bees, wasps and sawflies). Almost all knowledge about the genetics of sex determination in haplodiploid systems has been obtained from this group of insects, of which the honeybee, *Apis mellifera*, and the jewel wasp *Nasonia vitripennis*, have been investigated most intensively. Recently, the complete genome sequences of these two species have been published (The Honeybee Genome Sequencing Consortium 2006; Werren *et al*. 2010), increasing their worth as hymenopteran model organisms.

For a long time it has been known that multiple different sex-determining mechanisms exist within the Hymenoptera. Whiting (1933) was the first to show that sex determination in the wasp *Bracon* depends on the allelic state of a single locus. This mode of sex determination is called complementary sex determination (CSD) and has now been reported for over 60 hymenopteran species (Van Wilgenburg *et al*. 2006), including the honeybee. For recent reviews on the genetics and evolution of CSD within the Hymenoptera we refer to Van Wilgenburg *et al*. (2006) and Heimpel and de Boer *et al*. (2008). Beye and colleagues have unraveled much of the genetic regulation of CSD in the honeybee (Beye *et al*. 2003; Hasselmann *et al*. 2008; Gempe *et al*. 2009). In some hymenopteran groups, sex is not determined by CSD and these include the chaldidoid wasp *N. vitripennis*. In a number of recent studies Beukeboom and co-workers (Beukeboom and Kamping 2006; Beukeboom *et al*. 2007a,b; Kamping *et al*. 2007) have largely unravelled the genetic basis of sex determination in *Nasonia*. Here, we present the main findings as well as discuss some of the remaining unanswered questions.

*For correspondence. E-mail: L.W.Beukeboom@rug.nl.

Keywords. sex-specific splicing; imprinting; *doublesex*; *transformer*; Hymenoptera; *Nasonia.*

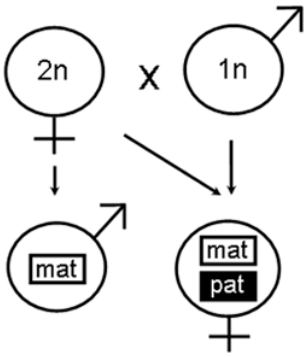

Figure 1. Haplodiploid sex determination. Females are diploid and produce haploid eggs. Males are haploid and produce haploid sperm. Unfertilized eggs develop into haploid males that only have a maternally inherited genome. Fertilized eggs develop into diploid females that have inherited a maternal and a paternal genome.

Early observations on sex determination in *Nasonia*

Absence of CSD in *Nasonia* has been known for a long time (Whiting 1967; Werren *et al*. 2010). Under CSD, matings between males and females that share a similar *csd* allele result in an increase of diploid homozygous individuals that are male. Although diploid males are known from *Nasonia*, they do not arise from inbreeding. Various alternative models of sex determination in *Nasonia* have been proposed and are discussed and reviewed in Beukeboom *et al*. (2007b). Whiting (1960) proposed that fertilization is required for female development. As an extension of this model it was proposed that sex determination in *Nasonia* depends on genomic imprinting (Beukeboom 1995; Beukeboom *et al*. 2007b). Under genomic imprinting, gene expression in the offspring depends on maternal or paternal inheritance. The genomic imprinting model states that a paternally inherited genome is required for female development. Dobson and Tanouye (1998) claimed to have found support for genomic imprinting sex determination in *N. vitripennis* but this was based on rejection of all alternative models at that time. Beukeboom and Kamping (2006) provided the first genetic evidence for a role of imprinting in *Nasonia* sex determination. Oliveira *et al*. (2009) and Verhulst *et al*. (2010a,b) largely unravelled the molecular genetic regulation of genomic imprinting sex determination (see below).

Sex-determination mutants

Much of the early information on *Nasonia* sex determination was obtained from mutants. Whiting (1960) studied a polyploid mutant which he found in his stock cultures. This mutant strain consists of triploid females that had low fecundity due to the production of predominantly aneuploid eggs, but as virgins also lay a small number of euploid haploid and diploid eggs (figure 2). Both haploid and diploid eggs typically develop into males. Diploid males are fully fertile and when crossed with diploid females yield triploid daughters. Eye-colour markers were used to distinguish between males of different ploidy (Beukeboom and Kamping 2006). Note that these *Nasonia* diploid males cannot originate from homozygosity under CSD.

The polyploid mutant has been instrumental for developing the genomic imprinting sex determination model (Poirié *et al*. 1992; Beukeboom 1995). Mated females produce two types of diploid offspring that develop into different sexes: unfertilized (uniparental) diploid eggs become males and fertilized (biparental) eggs become females (figure 2). As the fertilized eggs contain a maternal and a paternal genome copy, in contrast to the unfertilized diploid eggs, the paternal genome contributes differently to sex determination than the maternal genome. This was the first genetic proof of a role of genomic imprinting in *Nasonia* sex determination (Beukeboom *et al*. 2007b).

Kamping *et al*. (2007) described a gynandromorphic mutant that produced individuals that had both male and female morphology. Interestingly, such mutants were found at low frequency in several field-collected strains. As gynandromorphism was manifest in the offspring of mated as well as unmated females, it suggested that it was not a fertilization

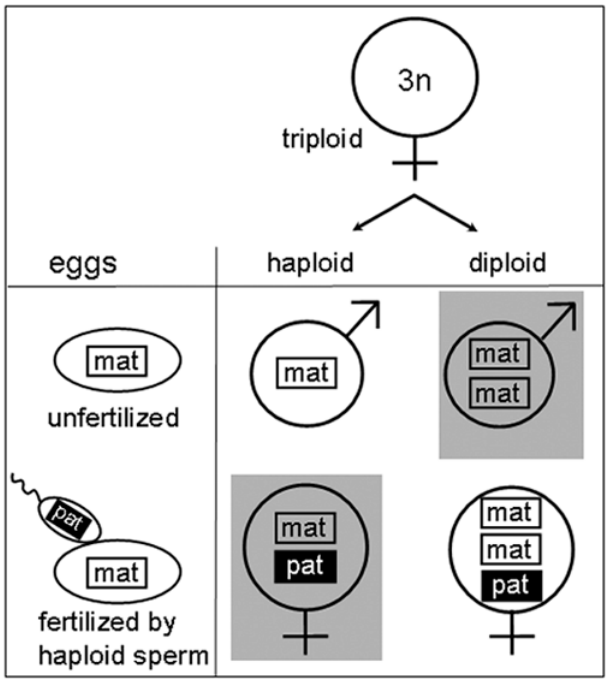

Figure 2. The polyploid mutant strain of *Nasonia*. Triploid females lay haploid and diploid eggs which develop into males if unfertilized. Fertilization of haploid and diploid eggs by haploid sperm results in diploid and triploid females, respectively. Unfertilized haploid and diploid eggs inherit (a) maternal genome(s) only, whereas fertilized eggs inherit a paternal genome in addition to the maternal genome(s). The data indicate a parental effect on sex determination because unfertilized diploid eggs develop into females, but fertilized haploid eggs into males (compare grey compartments).

syndrome. Morphological analysis and flow cytometry revealed that gynandromorphs were haploid rather than haplo–diploid mosaics, ruling out partial endoduplication as the cause (Beukeboom *et al*. 2007a). In one strain, the frequency and penetrance of gynandromorphism could be increased by selective breeding and this work resulted in the first example of haploid females in a hymenopteran species that, however, were almost completely sterile (Beukeboom *et al*. 2007a; Kamping *et al*. 2007). Gynandromorphs and females resulting from unfertilized haploid eggs in the gynandromorphic strain are indicative of a mutation in the sex-determination pathway. Functional genetic analysis suggested a temperature sensitive mutation in a maternal effect locus (*gyn1*) that produces a masculinizing product acting during a critical period in early embryogenesis as the cause of these exceptional (uniparental) females (Kamping *et al*. 2007). Alternatively, the mutation leads to partial derepression of a normally silenced *gyn1* that produces a feminizing product. This, however, is not easily reconciled with the observation that exposure to high temperatures increases the number of gynandromorph offspring, and would therefore require invoking another, temperature sensitive, genetic factor that regulates *gyn1*.

Genetic regulation of sex determination

Several decades of research into the genetic regulation of sex determination, originally focussed on *Drosophila melanogaster* and *Caenorhabditis elegans* (e.g. Cline and Meyer 1996), but later expanded to other species, has revealed evolutionary conservation of certain genes, but not others (Nöthiger and Steinmann-Zwicky 1985; Wilkins 1995; Saccone *et al*. 2002; Pane *et al*. 2005; Sánchez 2008). Sex determination occurs by a cascade of genes that are regulated in an hierarchical fashion. *Doublesex* is a basal functional switch gene that controls sexual differentiation into the female or male sex. It is sex-specifically spliced by *transformer*, whose splicing pattern in turn is regulated by a primary signal that is specific for the male and female sex. The primary signal is not conserved over a broad taxonomic range and varies from the X chromosome dose (e.g. *Drosophila*), to a dominant male determiner on the Y-chromosome (e.g. *Musca*) to a feminizing gene on the W-chromosome (e.g. *Bombyx*) in diploid insects with specialized sex chromosomes (Sánchez 2008). In the honeybee, the primary signal is established by complementation of the alleles at the *complementary sex determiner* locus (Beye *et al*. 2003). The other genes in the pathway, *transformer* (called *feminizer*, Hasselmann *et al*. 2008) and *doublesex*, are orthologs of similar genes in other insects.

Doublesex

The Nasonia Genome Project greatly facilitated the search for sex-determination genes. As expected, the basal gene *doublesex* is present in *Nasonia* (Oliveira *et al*. 2009) of which, a male-specific and female-specific splice form were identified, consistent with information from other insects. In gynandromorphs both splice forms were found confirming that *doublesex* is also involved in somatic sex determination in *Nasonia*. The first four exons of the mature splice form are identical between males and females. The *Nasonia* female splice form, however, is derived from two additional exons that are interrupted by an intron of 108 bp, whereas this whole region makes up one large fifth exon in males (figure 3). This exonic splicing pattern based on cryptic splice site selection is different from other insects where exon skipping is used to generate the male and female isoforms.

A phylogenetic analysis of the *doublesex* gene revealed that it clusters with *doublesex* of other insects. The gene has two distinct domains, a DNA binding domain (DM) and an oligomerization domain. Oliveira *et al*. (2009) aligned these two coding regions with similar *doublesex* sequences of eight other insects; four fly species, a beetle, a lepidopteran and the honeybee. The combined protein groups of *Nasonia* clustered together with that of the honeybee, the only other hymenopteran in the analysis. However, *Nasonia* did not cluster with *Apis* when only the DM domain was used, reflecting the amino acid divergence of the *Nasonia* DM domain. Further, a comparative analysis with the honeybee genome revealed microsynteny and provided further support for orthology of *Nasonia doublesex* (Oliveira *et al*. 2009).

Transformer

Screening of the *Nasonia* genome yielded a single gene with clear homology to *csd* and *feminizer* of the honeybee (Werren *et al*. 2010). The *Nasonia transformer* is composed of nine exons and contains two Arg/Ser-domains (SR-domains), of which one is located entirely in exon 1 and the second spans exons from 4 to 7. In exons 7 and 8, a proline (Pro) rich domain is present. Female-specific splicing retains only the first part of exon 2 and yields a single transcript encoding a full length protein of 405 amino acids, containing both SR domains and the Pro rich domain (figure 3). Three different transcripts can be found in male *Nasonia* due to cryptic splicing sites in exon two. All splice variants yield slightly different transcripts but they all contain several in-frame stop codons and encode truncated proteins containing only the first SR domain. Thus, the splicing pattern is sex-specific and resembles that of *transformer* genes in other species.

RNAi knockdown of *Nasonia vitripennis transformer* (*Nvtra*) in females in the late pupal stage resulted in complete sex reversal of fertilized eggs, causing them to develop into diploid males rather than females (Verhulst *et al*. 2010a,b). In haploid gynandromorphs, both male and female specific splice forms were observed and correlated with the degree of femaleness. This is reminiscent of doublesex splicing in gynandromoprhs and indicates that *transformer* is also involved in somatic sex determination in *Nasonia*. Further experiments revealed that *Nvtra* dsRNA injected mothers

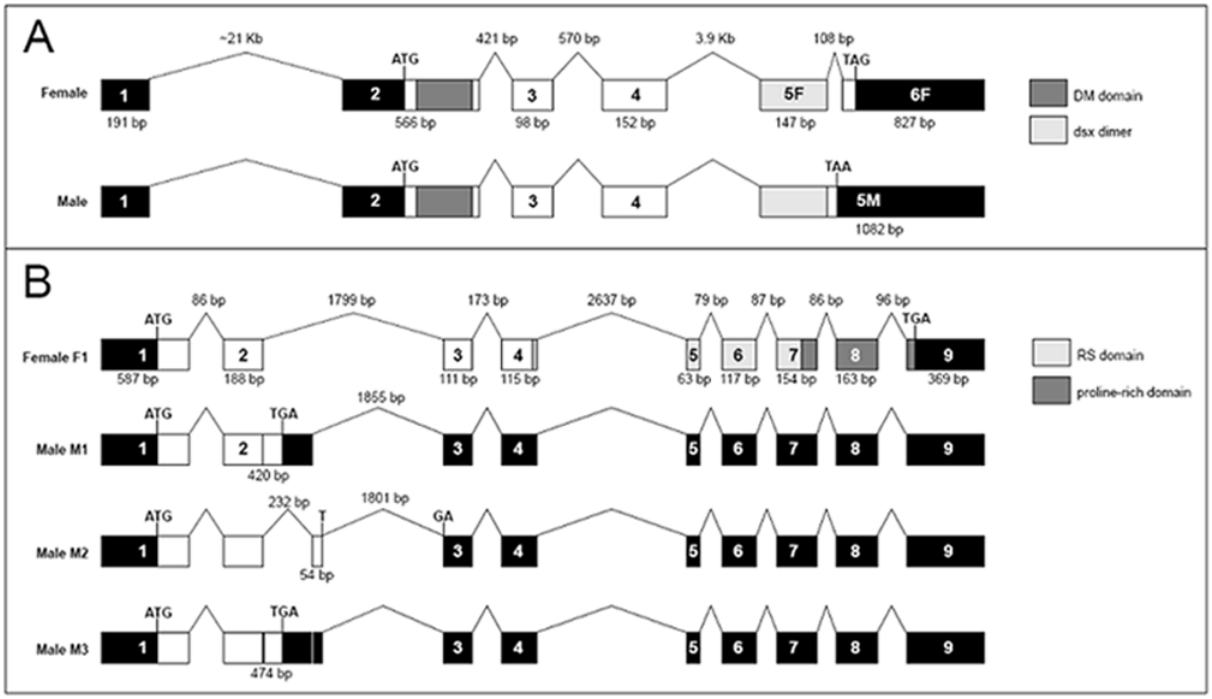

Figure 3. The *doublesex* (A) and *transformer* (B) gene of *Nasonia*. The male *doublesex* splice form contains five exons and the female splice form six exons. Exons 1 to 4 are present in both sexes, but exon 5 is complete in males but is spliced into two smaller exons in females. The *transformer* gene consists of nine exons in females, but cryptic splice sites in exon 2 yield longer transcripts but a truncated protein in males, due to in frame stopcodons in these transcripts. Note, the small difference in the presence of the most 3′ part of exon 2 in M1 versus M3.

provided lower amounts of *Nvtra* to the eggs, suggesting that female development of the fertilized egg depends on maternally provided female specific *Nvtra* mRNA. All in all, proper sex determination in *Nasonia* appears to rely on a zygotically active *transformer* gene that is autoregulated by maternally provided *transformer* transcript. Recently, Hediger *et al*. (2010) showed that in *Musca domestica* maternal provision of TRA is needed for processing of zygotic *tra*, as mutant females with suppressed germline expression of *tra* yielded only male offspring. Gempe *et al*. (2009) established a similar maternal provision of TRA for the hymenopteran *A. mellifera*. After the first description in *Ceratitis Capitata* (Pane *et al*. 2002, 2005), similar *transformer* autoregulatory loops have been proposed for the dipterans, *Bactrocera oleae* and *Lucilia cuprina* (Lagos *et al*. 2007; Concha and Scott 2009).

As haplodiploids have no specialized sex chromosomes that can provide the primary signal for sexual differentiation, such as a Y chromosome based male determiner or the X chromosome dose, the question was how males can develop in the presence of maternally provided *Nvtra*. Using crosses between two strains that differed in the presence of a deletion in a non-functional part of the *transformer* gene. Verhulst *et al*. (2010a) could show that a peak of zygotic transcription of *Nvtra* occurs after 7 h in fertilized embryos but not in unfertilized embryos. At this point it has not yet been resolved whether only the paternal allele of *Nvtra* is required for this expression peak maintaining the *transformer* autoregulatory loop, or whether a *trans* factor regulating the timely onset of zygotic *Nvtra* transcription is silenced in the maternally inherited genome. In other words, *Nasonia* females regulate the sex of the offspring by providing a feminizing effect by maternal input of *Nvtra* while at the same time preventing zygotic expression of *Nvtra* in haploid offspring. As only a paternally inherited genome copy results in timely transcription of zygotic *transformer*, sex determination in *Nasonia* most likely relies on maternal imprinting of a sex determination factor. As a result, unfertilized haploid eggs develop into males and fertilized diploid eggs into females, consistent with Whiting's original fertilization model (Whiting 1960).

In conclusion, *Nvtra* is part of the *Nasonia* sex-determining cascade and responsible for the sex-specific splicing of *Nvdsx* (figure 4). Sufficient levels of female-specific *Nvtra* transcripts are necessary to maintain the female-specific splicing pattern of *Nvtra* itself in the embryo. In addition, a paternally inherited genome appears to be required for this *Nvtra* autoregulatory loop, which constitutes a parent of origin or imprinting effect. The *Nasonia* system constitutes a novel mode of sex determination in insects that is unique in the way *transformer* is regulated. In *Nasonia* it is the mother that controls zygotic *transformer* expression, in contrast to all diploid insects studied thus far in which the paternal genome has a masculinizing effect by interrupting autoregulation of *transformer*.

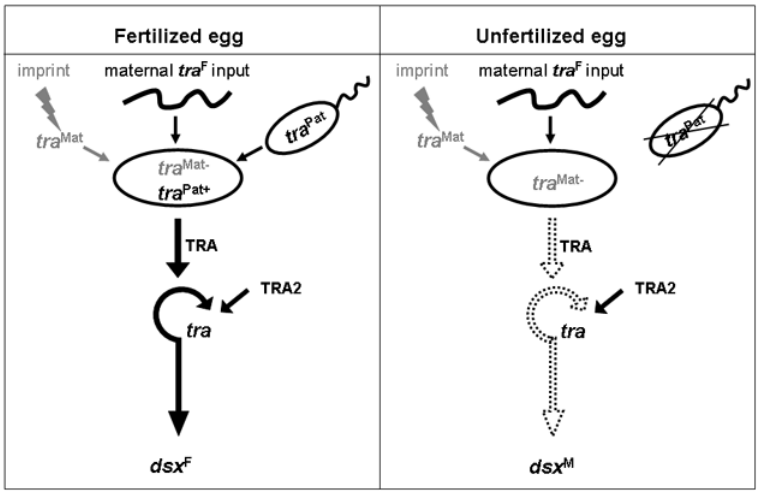

Figure 4. The genetic basis of sex determination in *Nasonia*. The cascade of genes consists of an unknown primary signal that imprints either the maternal *transformer* gene or a regulator of *transformer*. Maternal input of *Nvtra* RNA/protein autoregulates embryonic splicing of *tra* only if a paternally inherited genome is present (fertilized eggs) as *Nvtra* is transcriptionally silenced in embryos with only maternally derived genomes (unfertilized eggs). Sex-specific splicing of *Nvtra* regulates in turn the sex-specific splicing of *doublesex*.

Open questions

Although the basic pattern of sex determination in *Nasonia* has been unraveled, there remain a number of open questions that require further research. First, more details are needed about the primary signal. What is the nature of the imprinting process and which genes are involved? How does the paternal genome enable timely transcription of transformer in the zygote and how does it interact with the maternally contributed transformer?

Another issue concerns the evolution of different sex determining mechanisms within the Hymenoptera. CSD has often been considered as the ancestral mode of sex determination in this group (Cook 1993), but more comparative genomic studies and formal testing of a broader range of species from different hymenopteran taxa is needed for substantiating this claim. Interestingly, the *csd* gene appears to be absent in the *Nasonia* genome. A comparative genomic analysis revealed that *csd* in the honeybee resulted from a duplication of *feminizer*, but it also resides in a different region of the genome compared to *Nasonia*, likely due to a transposition in one of the two species. This suggests that CSD is not the ancestral state and calls for molecular studies of more basal groups, such as sawflies (Tenthredinoidea).

Another intriguing question is how transitions between sex determining mechanisms can occur. At this point, we still lack enough details about the phylogenetic distribution as well as the genetic regulation of complementary sex determination and genomic imprinting sex determination to determine how these two mechanisms are evolutionary related. The existence of different mechanisms in closely related groups suggests that alterations between systems can easily occur, but we do not understand how. For the case of *Nasonia*, an important next step would be to identify the *gyn1* locus responsible for gynandromorphism as this maternal effect locus is a candidate to be involved in the maternal imprinting process.

One of the main criticisms of the imprinting model of sex determination is that it would not explain sex determination in thelytokous Hymenoptera, because thelytoky is a form of uniparental all-female reproduction. Thelytoky is widespread among the Hymenoptera and has evolved multiple times from arrhenotoky (sexual reproduction) based on phylogenetic evidence. It refers to parthenogenetic development of females from diploid eggs. The criticism is that such eggs could not develop into females without a paternally inherited genome, as required by the imprinting model. Hence, independence from the requirement of a paternal genome for femaleness must have evolved in thelytokous species. Several cytological mechanisms of parthenogenetic egg development are known (Stenberg and Saura 2009). The main distinction is between apomictic (mitotic) and automictic (meiotic) processes, but for this discussion it is only important that in most cases egg diploidy is restored by fusion of two haploid nuclei during or after meiosis. The imprinting model can explain parthenogenetic female development by assuming that the imprint is put onto the maternal genome during oogenesis, but is not copied on to the replicated genome during the meiotic divisions of the egg. However, this remains a hypothesis until we know more about the biochemical details of the imprinting mechanism. Thus, for the moment it is unknown how sex determination functions

in thelytokous species that have no CSD. More knowledge about the genetic regulation of sex determining mechanisms in haplodiploids will help to explain the frequent evolution of thelytokous reproduction in haplodiploids organisms and whether this is due to different alterations of additional, hitherto unknown, sex determining mechanisms.

To date, the focus of haplodiploid sex determination research has been solely on the hymenopteran insects. CSD has been found in a number of hymenopterans, but the molecular genetic details have only been largely worked out for the honeybee (Gempe *et al*. 2009). It remains to be seen to what extent variations in the genetic regulation of CSD occur. Recent evidence for multiple loci involved in CSD (de Boer *et al*. 2008) adds an intriguing dimension to research into the genetic basis of CSD. Maternal effect genomic imprinting sex determination has thus far only been described from *Nasonia*. Although many hymenopterans are known to lack CSD, it is currently unknown how widely distributed this mechanism of sex determination is within the hymenopteran order and beyond. Moreover, how sex is determined in haplodiploid groups outside the order of Hymenoptera remains a completely unexplored field of research for the future.

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